micro technology with macro results

At Lawrence Livermore National Laboratory, engineers combine photonic technology and MEMS devices to produce hand-held biomedical test instrumentation.

By R. Mariella, Jr., A. Wang, R. Miles, P. Belgrader, S. Nasarabadi, W. Benett, J. Richards, P. Stratton, K. Venkateswaran, R. Langlois, R. Koopman, and F. Milanovich, Lawrence Livermore National Laboratory

Enabling the volume fabrication of low cost, miniature devices, MEMS technology has significantly advanced the design of portable biomedical instrumentation. Combined with advances in genome databases and bio-assays, the technology will allow researchers to build MEMS-based instruments that perform sample preparation as well as assays. Multiplex assays that look for numerous DNA targets in many different samples may change the face of medicine, speeding routine screening of blood samples for diseases like HIV, hepatitis, and herpes, as well as providing powerful tools for triage in emergency situations.

In the world of bio-assays, scaling is everything. Certain processes that are impractical in the macroscopic world, such as dielectrophoresis (DEP), merely needed miniaturization to make them useful. The assays themselves are often amenable to miniaturization, but they require a practical, reliable interface between real-world sample collection and a bio-MEMS instrument. Such techniques are part of sample handling and sample preparation, which are really on the critical path of success for the miniaturization of biomedical assays.

sample pretreatment

All materials have some polarizability, which, under the influence of an inhomogeneous electric field, can produce differential forces between components of a fluidic mixture. DEP exploits these polarizabilities to manipulate materials, allowing researchers to separate, concentrate, and sort cells and other particles in solution.[1-3]

The technique typically relies on an alternating voltage so that the charge on a molecule or particle is not a factor. The time-averaged dielectrophoretic force is a function of the gradient in the electric field, not a function of the applied voltage. To achieve high field gradients in macroscopic systems, one must apply large voltages, which cause the electrolysis of water, thereby interfering with most separation and manipulation procedures. For this reason, DEP in aqueous solutions is not compatible with macroscopic systems. It is, however, suitable for miniature fluidic systems.

Integrated sample preparation requires a high degree of functionality to enable biological or chemical analysis in miniaturized systems. Typically the cost of a part increases with its functionality, yet the danger of sample-to-sample contamination makes disposable parts attractive.

At Lawrence Livermore National Laboratory (LLNL; Livermore, CA), we sorted, mixed, and concentrated particles by remotely coupling acoustic energy into a glass or plastic chamber, combining high functionality with an inexpensive, disposable sample chamber.[4] Radiation pressure generated in the presence of a standing wave induces a pressure field that forces particles to collect in the nodes or antinodes of the standing wave. The acoustic force can transport particles as well as differentiate particles based on their size and material attributes. The bulk piezoelectric transducer can be used multiple times as the source of acoustic energy, while the sample chamber can be inexpensively processed and thrown away after one use.

pcr

The polymerase chain reaction (PCR) is an enzyme-based chemical reaction that manufactures copies of one or more selected regions of double-stranded DNA sequences (target sequences), contained within samples of DNA (substrates).[5] By

creating many copies of target sequences, PCR allows researchers to amplify the concentration of those sequences to a level that can be detected by techniques such as flow cytometry or spectrometry.

In the PCR process, which takes place in a complex reaction mixture, we raise the temperature of the reaction mixture to 95°C or 96°C, separating the double-stranded DNA into two single strands. Subsequently dropping the temperature to 60°C permits the DNA reagents, called primers, to hydrogen-bond to their complementary DNA sequence on the single-stranded DNA substrate.

Next, we increase the temperature to about 72°C, at which point the polymerase enzyme extends the primer by reading the single-stranded DNA target and covalently attaching one nucleotide at a time from the reaction mixture onto the 3' end of the primer. Each added nucleotide is complementary to its corresponding nucleotide on each target DNA sequence. The net effect is that the number of molecules of target DNA is doubled.

After this step, the entire process repeats in a method known as thermal cycling. When PCR works optimally, the number of copies of the target DNA sequence increases by 2^N, where N is the number of thermal cycles performed.

Purely an amplification process, PCR must be combined with detection techniques such as electrophoresis, *in situ* fluorescent detection, mass spectroscopy, hybridization, or flow cytometry to detect the presence of the target DNA sequence. In flow cytometry, molecules or particles in a flowing stream pass singly through an interrogating light beam, typically from a laser. As each particle intersects the beam, it generates elastic and inelastic scattering.[6] Photons scattered elastically though small angles provide information about the physical size of the particles, while photons scattered elastically at roughly 90° to the incident beam provide information about the convolution of particle size with particle granularity. Fluorescence also can be collected at 90°.

MEMS-based pcr

When compared with the original PCR procedure, which required electrophoresis to determine the presence of product(s), the real-time PCR/detection process has increased the sensitivity, specificity, and rapidity of the assay.[7,8]

The heart of our portable PCR instrument at LLNL is a micromachined silicon thermal-cycling chamber fabricated via anisotropic etching of single-crystal silicon, with two halves being bonded together (see figure 1). We coated each half with a thin-film resistor for the heating step in PCR; a thermistor at the bottom of the chamber provides feedback for temperature control. The etching produces a hexagonal opening along the length of the 2 × 6.5 × 23 mm sample chamber, which holds the polypropylene sample tube. The sample tube contains up to 25 µL of solution. Illumination at 480 to 490 nm from a high-brightness gallium nitride light-emitting diode (LED) passes through an optical filter, which eliminates longer wavelengths. Focusing optics direct the light through an aperture in the sidewall of the chamber where it passes through the sample tube to interact with the sample. An optically filtered silicon photodiode at the far side of the chamber captures fluorescence at 520 nm.

Our most recent fabrication efforts have yielded a low-thermal-mass chamber that requires less power for thermal cycling than did previous devices (see figure 2 on page 36), allowing us to fabricate a hand-held, battery-powered, real-time PCR instrument with four chambers. The chambers consist of two identical halves that are not bonded together but rather are spring-loaded to press against the sides of the plastic sample tube. The two-part chamber allows cooling air to pass more uniformly around the sample tube when the fan is operating and reduces the overall thermal mass of the system, both valuable attributes for a battery-powered instrument.

For the first field trials for our ten-chamber PCR instrument, we tested 320 unknown samples, 80 of which contained the vegetative bacterium, *Erwinia herbicola (E.h.)*. During the two-week trial, we evaluated the samples for concentration of *E.h.*, comparing them with results of overnight cell culture testing.

In the case of six out of 16 ($1/e$) of the samples whose nominal concentration was 10^2 bacteria/mL, the device detected no target DNA in the PCR reaction. This performance is consistent with sampling errors, as predicted by Poissonian statistics; such sampling errors did not appear at the higher *E.h.* concentrations. Essentially, there is a tradeoff between sample size and sensitivity: Smaller sample volumes yield a lower degree of sensitivity, giving false negatives for low concentrations.

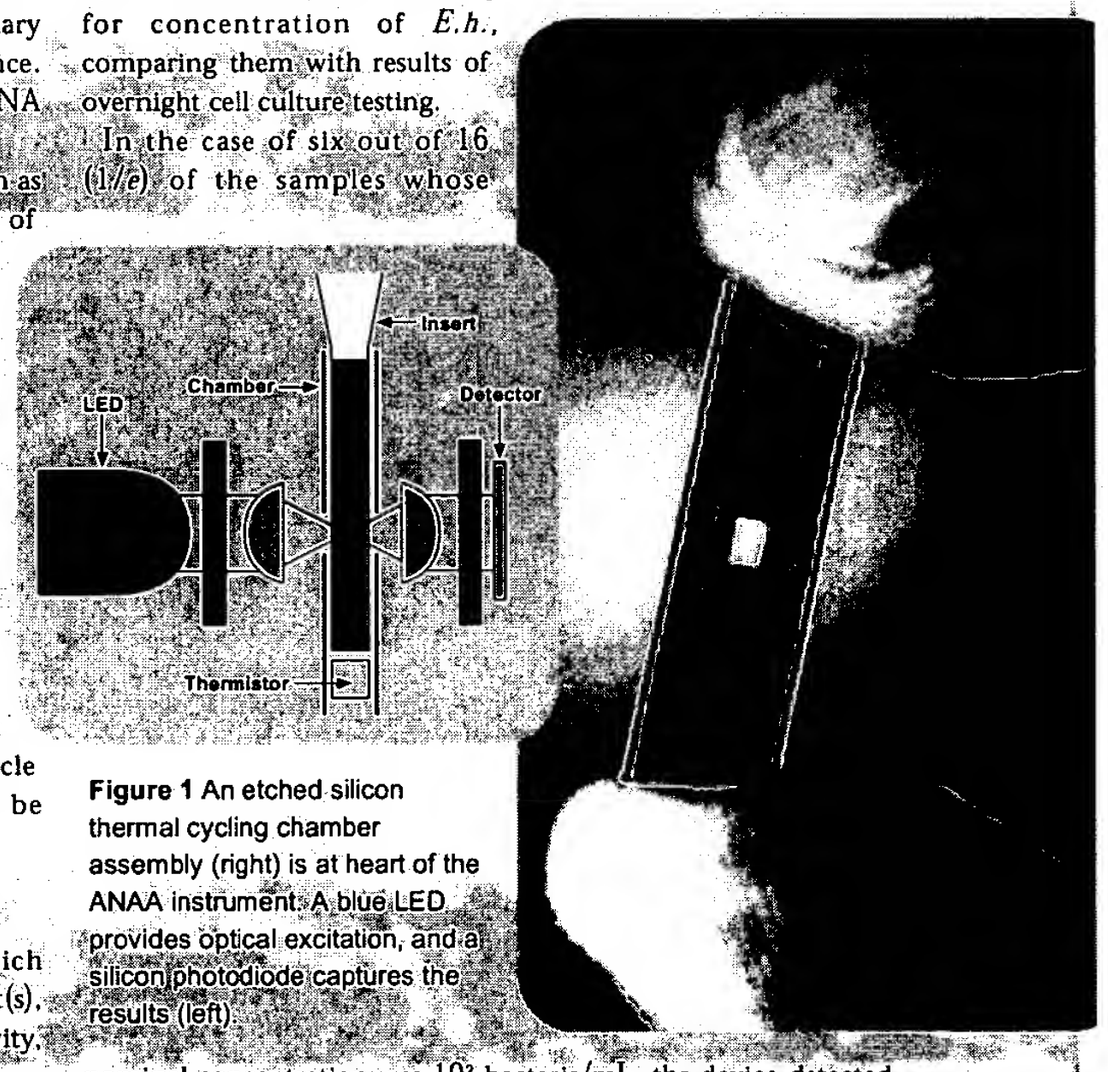

Figure 1 An etched silicon thermal cycling chamber assembly (right) is at heart of the ANAA instrument. A blue LED provides optical excitation, and a silicon photodiode captures the results (left).

multiplexed assays

Although real-time PCR is effective for single-target assays, there are considerable challenges to applying it to multiplexed assays, in which each distinct target sequence has a unique identifier. We will therefore discuss multiplex assays that do not attempt to perform real-time monitoring of the PCR, but rather that extract aliquots (or the entire reaction mixture) as part of the process in order to achieve multiplexing.

To increase the power of the procedure, researchers are now testing the use of PCR with other techniques, for example,

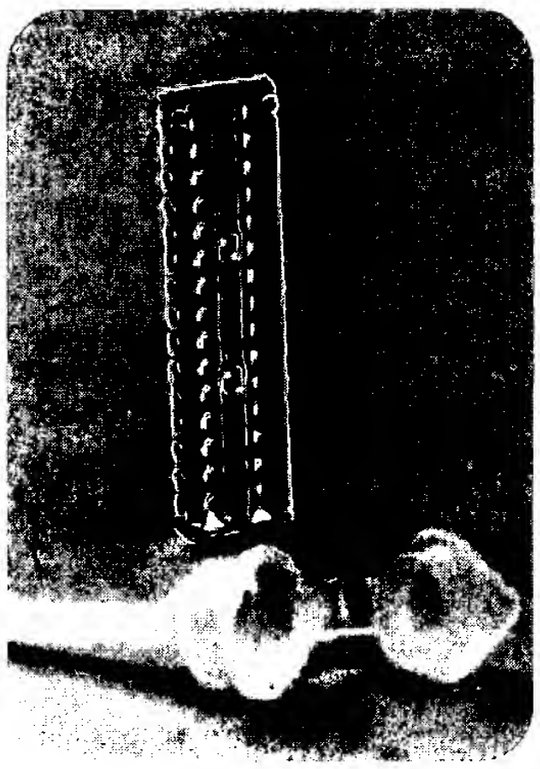

Figure 2 The silicon thermal cycling chamber for a hand-held PCR instrument consists of two identical halves, spring-loaded to press against the sides of the plastic sample tube. The chamber includes two windows to provide optical access to the sample tube, shown below the chamber.

combining *in-situ* PCR with flow cytometry.[9] The technique also has been integrated with electrophoresis and mass spectroscopy to increase assay throughput.

Rather than using fixed arrays to perform multiplex analysis of PCR reactions, we have been investigating the use of flow cytometry with liquid. Target DNA can be chemically attached to plastic beads that fluoresce when illuminated. The resultant biochemically derivatized beads can be analyzed using flow cytometry. Families of plastic beads from Luminex, Inc. (Austin, TX), distinguished from one another by their fluorescence at specific red and orange wavelengths, may be derivatized with different single-stranded DNA probes. In liquid array assays, each set of beads is hybridized with just one sample; then all such sets of beads are added together for flow analysis.

The approach allows the detection of up to 100 PCR products via hybridization, and multiple samples can be assayed for one or more targets in a single flow cytometric run. Such multiplexing is considerably more difficult to perform when hybridizing onto a fixed array.

The overall mixture is analyzed by flow cytometry, typically using a sandwich assay. In a sandwich assay, a single fluorescent label (yellow-green, for example) is chemically attached to a DNA probe that links to a portion of a target sequence of DNA, effectively making the target sequence fluorescent. Meanwhile, a probe for a different portion of the target DNA sequence is chemically attached to a set of beads. If the target sequence is present in the mixture, it attaches to both the DNA probe on the bead and to the fluorescent DNA probe from solution. As a result, when the bead passes through the excitation beam, it glows yellow-green in addition to its own fluorescence. **oe**

R. Mariella Jr., A. Wang, S. Nasarabadi, W. Benett, J. Richards, P. Stratton, K. Venkateswaran, R. Langlois, R. Koopman, and F. Milanovich are with Lawrence Livermore National Laboratory (LLNL). Ray Mariella can be reached at LLNL, PO Box 808, Mail Stop L-222, Livermore, CA 94551. Phone: 925-422-8905; fax: 925-422-2783; mariella1@llnl.gov R. Miles is now with K2 Optronics; P. Belgrader is with Cepheid Inc.

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References

The following is a partial set of references. For the complete reference list, please visit the oemagazine *Web site or contact Kristin Lewotsky at kristinl@oemagazine.com.*

1. Pohl H; Dielectrophoresis, *Cambridge Univ. Press, Cambridge, 1978.*

2. M. Talary, et al., Medical and Biological Engineering and Computing, 1995, 33:235-7.

3. Y. Huang, et al., J. Hemat. Stem Cell Res, 1999, 8:481-90.

4. A. Wang et al., 2000 ASME International Mechanical Engineering Congress and Exposition.

5. K. Mullis et al., Cold Spring Harbor Symposia on Quantitative Biology, 1986, 51:263-73.

6. See, for example, Shapiro HM. Practical Flow Cytometry, 2nd ed. *Wiley-Liss, New York, 1988, or more recent edtions.*

7. R. Higuchi, et al., Bio/Technology, 1993, 11:1026-1030.

8. P. Belgrader, et al., Science, 1999, 284: 449-50.

9. M. Iannone, et al., Cytometry, 2000, 39:131-140.

Shedding light on PCR

Biomedical instrumentation was not always as sophisticated as the Lawrence Livermore National Laboratory (LLNL) project. In the early '90s, Russell Higuchi and Robert Watson of Roche Molecular Systems (Emeryville, CA) were developing a method for real-time detection of the polymerase chain reaction (PCR), and Ray Mariella pitched in to help with a makeshift setup that was surprisingly successful.

At the time, researchers detecting PCR products placed a sample in agarose gel with an ethidium-bromide dye that worked its way into the DNA helix. Under green excitation, the dye fluoresced at around 600 nm, indicating the presence of DNA. The whole process required several hours to complete.

Higuchi and Watson had already successfully skipped the agarose-gel step and instead were putting the dye directly into the PCR product. "One of them decided to try a real-time version of the process," Mariella says. "They wanted to put the ethidium bromide in the tube before running the PCR and try to look for fluorescence while it was developing."

The problem was that they weren't quite sure how to set up the apparatus. M. Allen Northrop, an LLNL colleague who was involved with Higuchi and Watson at Roche, suggested Mariella as the solution. "Allen knew I had set up a lot of apparati of this type and said, 'Why don't you come along and help?'"

The researchers had set aside a large closet at Roche for the work, with an old lantern slide projector as a source. Mariella filtered the lantern output with a piece of green plastic film and steered the beam into the thermal cycler holding the PCR sample tubes. For a detector, Higuchi and Watson had borrowed a charge-coupled-device (CCD) camera. Mariella set it up to view the reaction cells, filtering the input with a red plastic film. "They could shine green light into the reaction while it was running and look at what was happening with the CCD camera," Mariella says.

He admits quite frankly that he hadn't expected the experiment to work. "I was mostly worried about melting the plastic," he says. "The lantern slide projector was very powerful—I'd guess 500 W or so. The output lens had to be three inches across."

"The whole thing was very crude, of course. In fact when we turned on the lantern projector and were stuck in that little closet, you could smell the ozone. But before too long you could see the cells were starting to glow. It worked, and that was quite exciting."

—Kristin Lewotsky